VOLUME 29 MARCH 1951 NUMBER 3

Canadian Journal of Technology

Editor: G. A. LEDINGHAM

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF TECHNOLOGY

This was formerly *Section F, Canadian Journal of Research.* The change to the new name took place January 1, 1951. The CANADIAN JOURNAL OF TECHNOLOGY is published twelve times annually.

The CANADIAN JOURNAL OF TECHNOLOGY is published by the National Research Council of Canada under the authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research. Matters of general policy are the responsibility of a joint Editorial Board consisting of members of the National Research Council of Canada and the Royal Society of Canada.

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Canadian Journal of Technology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 29 MARCH, 1951 NUMBER 3

PRODUCTION AND PROPERTIES OF 2,3-BUTANEDIOL

XXXV. PHOSPHATE DEFICIENCY AS A FACTOR IN THE PRODUCTION OF 2,3-BUTANEDIOL FROM SUGAR-BEET MOLASSES[1]

BY D. MURPHY,[2] D. W. STRANKS,[3] AND G. W. HARMSEN[3]

Abstract

Bacillus polymyxa, Aerobacter aerogenes, Bacillus subtilis, and *Serratia marcescens,* when cultivated on molasses media were stimulated in growth and in butanediol production by adding natural organic substances such as wheat bran, vegetable juices or extracts, yeast extract, or corn-steep liquor. The most important beneficial factor in all these substances was the phosphorus content. It was necessary to add the phosphorus-containing compounds as orthophosphate, or in a form that would readily yield orthophosphate. Most of the sugar-beet molasses tested were found deficient in phosphates. The natural organic substances contained, in addition to phosphate, some minor factors that produced a small but noticeable stimulation of the fermentation in molasses media. These factors have not been identified.

Introduction

The extensive work done on production of 2,3-butanediol by fermentation has been mainly confined to media prepared from pure sugars or to starch or grain mashes. An exhaustive review of the literature on this subject was recently published by Ledingham and Neish (12). Very little work has been done on the use of molasses in this fermentation. During the years 1929–1936, patents were granted to Kluyver and Scheffer (8), and Scheffer (15) in which they claimed the successful fermentation of molasses to butanediol. The yields ranged from 30% to 50% based on the original sugar, and the fermentation time was 36 hr. In 1944, Christensen (1, 2) took out patents on the fermentation of molasses for butanediol production. In the same year, Torres and Frias (14) published a short study on the fermentation of cane molasses. In 1947, Freeman and Morrison (4) published a more extensive study, using several different types of molasses but only one organism, *Aerobacter aerogenes.*

Most of the published work on this use of molasses appeared in patent claims, which presented the subject superficially. It was decided, therefore,

[1] *Manuscript received September 12, 1950.*
Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as Paper No. 103 on the Industrial Utilization of Wastes and Surpluses and as N.R.C. No. 2327.
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to conduct a careful investigation as an extension of our work on 2,3-butanediol. The need for this research was obvious since the production of butanediol can become profitable only if a cheaper raw material than starch or grain mashes can be used.

Our first report on the utilization of molasses media was presented by Simpson and Stranks (16). They studied practically every factor that could affect the fermentation by *Bacillus polymyxa* and evolved an adequate and very cheap procedure. They concluded that sugar-beet molasses was an excellent raw material for the butanediol fermentation. One of their most striking results was the very strong influence of added organic substances such as yeast extract, malt sprouts, whole wheat, or wheat bran.

The main object of the present work was to determine the exact constituents of organic stimulants that were responsible for accelerating the fermentation and increasing the yields of metabolic products. Production of 2,3-butanediol from sugar-beet molasses by other groups of organisms was also investigated: the organisms were *Aerobacter aerogenes*, *Bacillus subtilis*, and *Serratia marcescens*.

Experimental

Materials and Methods

Organisms

The following organisms were used during the work: *Bacillus polymyxa*, strains C3(2), C42(3)E13, and C38(2), all local isolates; *Aerobacter aerogenes*, strain M148, a local isolate, and 199 from the Northern Regional Research Laboratories, Peoria, Ill.; *Bacillus subtilis*, strain 200, a local isolate, and B44 (originally No. S8, Ford strain, from Dr. Gunsalus, Cornell University); *Serratia marcescens*, strain S25 (from Dr. Breed, New York Agricultural Station, Geneva, N.Y.), and strain S16 (from Dr. Eagles, University of British Columbia).

Taxonomically, Bergey's Manual of Determinative Bacteriology, sixth edition, was followed. The complete identification of the strains was, therefore, *Bacillus polymyxa* (Prazmowski) Migula, *Aerobacter aerogenes* (Kruse) Beyerinck, *Bacillus subtilis* Cohn emend. Prazmowski, and *Serratia marcescens* Bizio. During the course of the work the organisms were kept on molasses agar slopes under mineral oil.

Medium Used

In all the work described in this paper the basal medium used was 10% molasses, which was recommended as optimum by Simpson and Stranks (16). This gave an initial sugar concentration of approximately 5%. Glacial acetic acid was added to bring the pH to 5.7. During sterilization this rose to 6.3, which was close to the optimum for the fermentation. The usual sterilization period was 15 min. at 15 p.s.i. No neutralizing or buffering agent was added

because the natural buffering capacity of the medium prevented any appreciable shift in reaction during fermentation. Two samples of molasses were used almost exclusively in the investigation, Chatham 1946 and 1947, both supplied by the Canada and Dominion Sugar Company Limited, Chatham, Ont.

Fermentations

In most experiments the amount of medium used was 100 ml., which was dispensed in 500-ml. Erlenmeyer flasks. They were inoculated with 0.5 to 1.0 ml., and in some series with 10 ml. of the organism grown for 24 hr. on the same medium. During the fermentation the flasks were shaken on a Gump rotating shaker at 100 r.p.m., or on a reciprocating shaker at 90 strokes per minute. In these experiments in which carbon dioxide evolution was measured the temperature was 30°C., in the others, 35°C.

Cotton-wool plugs were used in most of the experiments. When the cultures had to be sampled during the fermentation, flasks with special ground-glass stoppers were used. About 10 liters of carbon-dioxide-free air was passed through these flasks every day and the evolved carbon dioxide was determined by absorption in sodium hydroxide solution.

Analyses

Butanediol was determined by the butanol extraction and periodate oxidation method described by Leslie and Castagne (13). Ethanol was determined by distillation and dichromate oxidation. Initial and residual sugars were determined by the method of Somogyi and Shaffer as revised by Underkofler *et al.* (18), or by the Lane–Eynon method (10). Carbon dioxide evolved was absorbed by sodium hydroxide and was determined by titration using bromthymol blue as indicator and adding excess barium chloride.

Results

Stimulation by Various Organic Substances

The findings of Simpson and Stranks (16), that adding organic substances increased speed of fermentation and yield of metabolic products by *B. polymyxa*, was first confirmed. Results are shown graphically in Fig. 1. The small initial amount of butanediol was introduced in the inoculum. The apparent increase in residual sugar after 52 hr. was due to acetoin, which reacted with the reagents used to determine the sugar content. Certain organic substances such as soil and manure extract had only an insignificant effect on the fermentation.

Bran and other stimulants had a beneficial effect on the fermentation in very small amounts, pointing to a deficiency in the molasses. Katznelson (5, 6) and Katznelson and Lochhead (7) have shown clearly that *B. polymyxa* required organic growth-substances for optimum development and efficient

butanediol formation. Other workers have confirmed these findings (3, 9, 11, 16). Several vitamins, casein hydrolyzate, and two mixtures of amino acids were therefore tested. In addition, to test whether added minerals had a

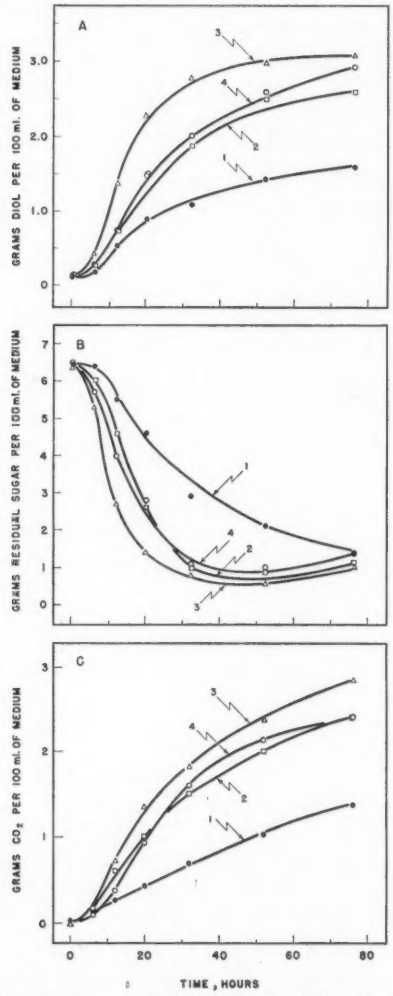

FIG. 1. *Effect of adding various organic stimulants to a 10% molasses medium on diol yields, sugar consumption, and carbon dioxide evolution in fermentations by B. polymyxa strain C42(3) E13. Curves numbered: (1) Basal medium. (2) Basal medium plus 0.11% bran. (3) Basal medium plus 0.3% yeast extract. (4) Basal medium plus 5% tomato juice.*

stimulating effect on the fermentation, molasses ash was added to one series of flasks. Effects of the various additions are shown in Table I. While some of the vitamins and the amino acid mixtures had an effect on the fermentation,

TABLE I

VARIATION IN BUTANEDIOL PRODUCTION FROM 10% MOLASSES MEDIUM BY *B. polymyxa* STRAIN C42(3)E13 IN THE PRESENCE OF VARIOUS ADDITIVES

(Diol yield expressed in grams per 100 ml.)

Substance added	Diol yield				
	12 hr.	20 hr.	32 hr.	52 hr.	76 hr.
None	0.5	0.75	1.1	1.5	1.7
0.11% bran	0.9	1.4	2.1	2.2	2.25
0.2% casein hydrolyzate*	1.0	1.7	2.3	2.55	2.50
Biotin (5 μgm. per l.)	0.45	0.7	1.0	1.5	1.9
Thiamine (200 μgm.)	0.5	0.75	1.1	1.4	1.6
Pyridoxin (200 μgm. l.)	0.55	0.75	1.1	1.65	2.0
Nicotinic acid (500 μgm. l.)	0.5	0.8	1.5	1.8	2.0
Ascorbic acid (500 μgm. l.)	0.5	0.7	1.2	1.9	2.1
All vitamins	0.6	1.1	1.7	1.9	1.7
8 amino acids **	0.6	0.9	1.2	1.7	—
18 amino acids***	0.5	0.9	1.2	1.8	—
Ash from 10 gm. molasses	0.8	1.45	2.0	2.25	2.35

* *"Vitamin-free" SMACO. General Biochemicals, Inc., Chagrin Falls, Ohio.*

** *dl-alanine, l(+)-arginine, dl-aspartic acid, l(−)-cystine, l(+)-glutamic acid, glycine, l(−)-histidine, dl-isoleucine.*

*** *Amino acids in ** plus l(−)-leucine, dl-lysine, dl-methionine, dl-phenylalanine, l(−)-proline, dl-serine, dl-threonine, l(−)-tryptophane, l(−)-tyrosine, and dl-valine. All added at the rate of 20 mgm. in 100 ml. of medium.*

it was not as marked as that shown by bran, casein hydrolyzate, or molasses ash. This experiment showed that stimulation was due to mineral constituents of the added substances. This finding is borne out in Fig. 2, which shows that the effect of adding bran, an aqueous extract of bran, and bran ash to a series of fermentations. Since molasses ash stimulated the fermentation it is reasonable to assume that the responsible element or elements were mainly in a bound or inaccessible form in the molasses.

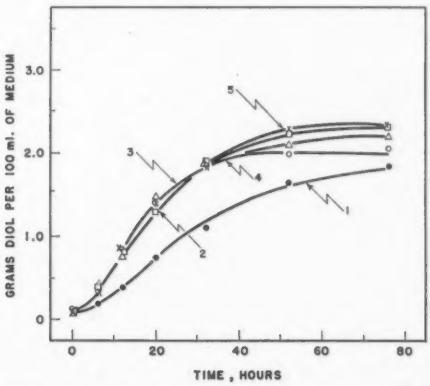

FIG. 2. *Yields of diol from B. polymyxa strain C42(3)E13 on a 10% molasses medium with various additions. Curves numbered: (1) Basal medium. (2) 0.11% bran. (3) Extract of bran. (4) Residue from bran in curve (3). (5) Ash of 0.5 gm. bran.*

All work reported so far was done with strain C42(3)E13 of *B. polymyxa*. At the same time work was also being done with another butanediol-producing organism, *Aerobacter aerogenes* strain M148. It was shown that, if 0.5% by weight of corn-steep liquor or its ash was added to a 10% molasses medium on which this organism was grown, there was a marked increase in the amount of butanediol formed. In one such experiment, after a 24 hr. fermentation, the control flask had 0.4% diol, the flask with added corn-steep liquor 1.9%, and that with corn-steep liquor ash, 1.8%. The slightly higher figure obtained in the flask with added corn-steep liquor over that with the ash was probably due in part to fermentable substances in the corn-steep liquor.

Determination of the Nature of the Stimulatory Factor

Published analyses on the constituents of corn-steep liquor showed that the liquor contained upwards of 20 elements (14). Twenty-three elements were tested separately in an effort to find the one responsible for the improved fermentation by *A. aerogenes* in the presence of corn-steep liquor.* None of the salts tried showed any effect on the fermentation. A similar test with even more elements, performed with *B. polymyxa* as the test organism, gave completely negative results. Spectrographic analyses were made of the ashes of molasses, corn-steep liquor, and bran, using sensitive plates. The molasses was found to contain iron, zinc, aluminum, magnesium, molybdenum, copper, sodium, potassium, calcium, chromium, boron, silicon, manganese, vanadium, and lanthanum. Bran and corn-steep ashes contained all these elements and, in addition, gave lines for nickel, silver, and phosphorus. To test whether any of these three last elements was responsible for stimulating the fermentation, various combinations of these elements were added to the basal medium. The contents of the flasks were analyzed after a 24-hr. incubation, with the results shown in Table II. The material that exercised the beneficial influence

TABLE II

STIMULATION OF DIOL PRODUCTION OF *A. aerogenes* M148 BY THE ADDITION OF CORN-STEEP LIQUOR AND VARIOUS IONS TO A 10% MOLASSES MEDIUM

(Diol expressed as grams per 100 ml. of medium; fermentation time, 24 hr.)

Additive	Diol produced
None	0.4
0.5% corn-steep liquor	1.9
Corn-steep liquor ash	1.8
P†	1.9
Ag + Ni	0.4
P + Ag	1.9
P + Ni	1.9
Ag + P + Ni	2.0

† *Phosphorus added as dibasic sodium phosphate, silver as silver nitrate, and nickel as nickel sulphate, each at a concentration of 0.01%.*

* *Those tested were Al, Fe, Co, Cd, Cs, Ba, Sb, Ga, Cu, Mg, Ce, Zn, Mo, Ag, Sr, Se, B, W, Na, Li, K, Bi, and I. The elements under test were added as 0.005% by weight to the basal medium.*

on the fermentation was the phosphate ion. This confirmed the opinions of Kluyver and Scheffer (8), Scheffer (15), and Freeman and Morrison (4), all of whom claimed successful fermentations only when phosphates were added to the medium. Simpson and Stranks (16) and Christensen (1, 2) added organic stimulants to their molasses media and it is understandable that these workers overlooked the need for phosphorus compounds.

Analyses of the molasses used in these laboratories showed that it contained 0.03% phosphorus as phosphorus pentoxide. It is probable that it volatilized during ashing and hence did not appear in the spectrographic analysis of the ash. It can be assumed that most of the phosphorus in the molasses was inaccessible to *A. aerogenes* since 0.004% phosphorus pentoxide added as sodium phosphate had a much better effect on the fermentation than the 0.003% phosphorus pentoxide originally present in the 10% molasses medium.

The effect of phosphate addition was also tried with *B. polymyxa* strain C42(3)E13. Dibasic sodium phosphate dihydrate was added to the medium to give a concentration of 0.25% phosphorus pentoxide. This medium was compared with the standard medium (0.11% bran) and with the basal medium. Fig. 3 shows the results obtained. Only the curves for butanediol production are shown; those for the other metabolic products followed the same trend very closely. As with *A. aerogenes* M148, this strain of *B. polymyxa* showed that deficiencies in the control medium were accounted for almost entirely by phosphorus. It will be noted, however, that the simultaneous addition of phosphate and bran to the medium gave slightly higher yields than when they were added separately (Curve 4 compared with Curves 2 and 3). This shows that there is in bran some growth factor, other than phosphorus, which gives a slight extra effect.

Effects of Added Phosphate on Diol Production by Other Organisms

Since such taxonomically remote organisms as *Bacillus polymyxa* and *Aerobacter aerogenes* responded so markedly to the addition of phosphate to molasses media, two other organisms, *Bacillus subtilis* and *Serratia marcescens*, were tested for their need for phosphate when grown on the same media. No report has so far appeared on the ability of these organisms to produce butanediol from molasses media or on their need for added phosphate. More than one strain of each organism was tried. The results are shown in Table III. It is seen that *B. subtilis* produced a relatively small amount of butanediol in the absence of phosphate. The addition of 0.1% dibasic sodium phosphate to the molasses, however, almost completely overcame its deficiencies as a medium for this organism. *S. marcescens* showed scarcely any diol production on the basal medium but was much improved when phosphate was added. The effect of added phosphate on the butanediol fermentation is apparently not specific for *B. polymyxa* or *A. aerogenes* but seems to be a general phenomenon with media made from the molasses under investigation.

TABLE III

THE PRODUCTION OF DIOL BY STRAINS OF *B. subtilis* AND *S. marcescens* ON 10% MOLASSES MEDIUM
(PO_4 ion added as 0.1% $Na_2HPO_4.2H_2O$; diol yields, gm. per 100 ml. medium)

Organism		Addition of phosphate	24 hr.	48 hr.	72 hr.
B. subtilis	B44	−	—	—	0.7
"		+	1.6	1.6	—
"	B200	−	—	—	0.95
"		+	1.45	2.0	—
S. marcescens	S25	−	—	—	0.05
"		+	0.5	1.0	—
"	S16	−	—	—	0.05
"		+	0.6	1.25	—

Optimum Concentration of Phosphate

For *B. polymyxa* strain C3(2) the dibasic sodium phosphate content of the medium was varied in steps from 0.002 to 0.3% by weight. The products were analyzed after 24 and 48 hr. for butanediol (Table IV). The lowest concentration of sodium phosphate that exerted the optimum effect on the fermentation in 24 hr. was between 0.005% and 0.01%; in 48 hr. it was around

TABLE IV

THE EFFECT OF VARYING AMOUNTS OF DIBASIC SODIUM PHOSPHATE ON THE FERMENTATION OF A 10% MOLASSES MEDIUM BY *B. polymyxa* STRAIN C3(2)
(Diol yield gm. per 100 ml. medium)

Concentration of $Na_2HPO_4.2H_2O$, %	24 hr.	48 hr.
Control	0.35	0.95
0.002	0.65	1.55
0.005	0.85	1.65
0.01	1.1	1.7
0.02	1.1	1.7
0.05	1.15	1.65
0.1	1.15	1.65
0.2	1.1	1.6
0.3	1.05	1.65

0.002%. This figure corresponded to the addition of phosphorus as pentoxide of only 0.001%. The molasses in use had 0.03% phosphorus as pentoxide, consequently the 10% molasses solution being fermented had 0.003% phosphorus as pentoxide. It follows, therefore, that most of the original phosphorus in the molasses must have been inaccessibly bound in a very resistant compound.

Similar quantitative results were later obtained with three other organisms: the minimum concentration of additional dibasic sodium phosphate dihydrate

was: for *A. aerogenes* about 0.005%, for *B. subtilis* also about 0.005%, and for *S. marcescens* about 0.02%.

Effects of Various Forms of Phosphate

On *A. aerogenes* strain 148, the effect of other phosphate-containing compounds (phosphoric acid, ammonium phosphate, and calcium superphosphate) was compared to that obtained with dibasic sodium phosphate. Each compound was added to give the same phosphorus pentoxide concentration in the medium as was given by 0.1% dibasic sodium phosphate. This experiment was of more than academic interest since the cheapest compound would be important on pilot-plant and commercial scales. It was found that all the compounds tested gave the same high yield of diol in 24 hr. and that the cation was of no importance.

In all experiments described in this paper so far the phosphorus-containing salt which was added to the medium was in the form of salts with the orthophosphate or PO_4^{---} radical. To test whether other forms of phosphorus would influence the fermentation, the following compounds were added to a series of flasks: "Calgon" or sodium hexametaphosphate ($Na_6(PO_3)_6$), sodium hypophosphite ($NaH_2PO_2.H_2O$), sodium pyrophosphate ($Na_4P_2O_7$), sodium phosphite ($Na_2HPO_3.5H_2O$), sodium metaphosphate ($NaPO_3)_n$, and lecithin. All were added in the concentration of 0.1 gm. per 100 ml. of medium except lecithin, which was added at the rate of 0.2 gm. per 100 ml. because of its lower phosphorus content. *B. polymyxa* strain C3(2) was tested for its reaction to these compounds. The results are set out in Table V.

TABLE V

THE EFFECT OF VARIOUS PHOSPHORUS-CONTAINING COMPOUNDS ON THE PRODUCTION OF DIOL BY *B. polymyxa* FROM 10% MOLASSES MEDIUM

(Diol yield, gm. per 100 ml. medium)

Compound added	24 hr.	48 hr.
None	0.4	0.8
Dibasic sodium phosphate	1.1	1.9
"Calgon"	1.15	1.65
Sodium pyrophosphate	1.45	1.85
Sodium phosphite	0.3	0.7
Sodium hypophosphite	0.3	0.65
Sodium metaphosphate	1.15	1.7
Lecithin	0.7	2.0

All the salts tested, except sodium phosphite and hypophosphite, gave a marked increase in yield of diol over the control. Sodium pyrophosphate, "Calgon", and sodium metaphosphate probably changed to the orthophosphate or PO_4^{---} form, at least to a degree sufficient to affect the fermentation. Lecithin may have disintegrated also to form orthophosphate. Both sodium hypophosphite and phosphite inhibited the fermentation noticeably: these

salts may be regarded as being derived from phosphorus trioxide, while all the others are derived from the pentoxide.

Effects of Phosphate on Various Molasses

In all the experiments described so far the molasses used was 1947 Chatham molasses of the Canada and Dominion Sugar Company, Ltd. Four other molasses, Alberta Steffen, Alberta non-Steffen, Manitoba, and Chatham 1946, were then tested. Dibasic sodium phosphate was added to media prepared from the various molasses at a concentration of 0.1% by weight. Two organisms, *B. polymyxa* strain C3(2) and *A. aerogenes* strain 148, were used. The results obtained are shown in Table VI. In four of the molasses, Alberta

TABLE VI

YIELDS OF DIOL FROM VARIOUS SAMPLES OF MOLASSES, WITH AND WITHOUT ADDED DIBASIC SODIUM PHOSPHATE

(Yield of diol, gm. per 100 ml.)

Molasses used	Addition of phosphate	*B. polymyxa*			*A. aerogenes*
		24 hr.	48 hr.	72 hr.	24 hr.
Alberta Steffen	−	0.7	1.35	1.45	0.7
"	+	1.2	1.75	1.75	1.8
Alberta non-Steffen	−	1.0	1.6	1.5	1.45
"	+	1.55	1.6	1.6	1.75
Manitoba	−	0.5	1.0	1.0	0.45
"	+	0.95	1.6	1.5	1.8
Chatham 1947	−	0.7	1.45	1.7	—
"	+	1.65	2.0	2.0	—
Chatham 1946	−	—	—	—	0.6
"	+	—	—	—	1.75

Steffen, Manitoba, Chatham 1947, and Chatham 1946 a very marked increase in butanediol production, in growth, and in all other activities was shown by both organisms in the presence of added phosphate. For Alberta non-Steffen molasses, however, only a small effect was shown. Growth and activity of the organisms on this molasses were fairly good without added phosphate. In 48 hr. the sample without added phosphate produced as much butanediol as the sample to which phosphate was added. This type of molasses is probably richer in available phosphorus than the other samples.

Fermentation in Six-Liter Amounts of Medium

In all the experiments described the amount of medium used was 100 ml. in 500 ml. Erlenmeyer flasks. This gave shallow, well aerated layers. At the end of the investigation the stimulating effect of added phosphate was also checked in fermentations conducted with six-liter aliquots of medium in nine-liter Pyrex bottles. The bottles were shaken and aerated during the fermentation. Sodium phosphate showed the same stimulating effect as it did in the smaller flasks. All the organisms tested gave similar results under these conditions.

Discussion

Analysis of the molasses used in most of these experiments showed that it contained three times as much phosphorus as was necessary to support an adequate fermentation. Nevertheless, all the experiments described in this paper show that the molasses seemed to be deficient in phosphorus compounds. One explanation is that most of the phosphorus in the molasses was unavailable to the organisms, being tightly bound in organic or inorganic compounds. This view is strongly supported by the fact that molasses ash added to a molasses medium stimulated the butanediol fermentation. The ashing released the phosphorus from its bound form.

Not only diol formation, but the entire metabolism of the organisms is stimulated by phosphate addition. In fact, the beneficial action can first be noticed in accelerated cell division and growth of the cell population. It follows that the most likely role that phosphate plays is in entering into the various phosphorylation cycles. This is borne out by the necessity of adding the phosphorus in the orthophosphate (PO_4^{---}) form or in a form that would change easily to orthophosphate. Pyro- and metaphosphate change easily and benefit the fermentation, while phosphite and hypophosphite, which do not change, affect the fermentation adversely.

An organism like *B. polymyxa*, which cannot grow on synthetic media because of the shortage of vitamin-like substances, can grow nearly optimally on molasses media with no other addition than that of phosphate, proving that molasses is comparatively rich in organic growth-substances. This conclusion, however, is tenable only where the major growth-factors are concerned, since a molasses medium with added phosphate did not prove to be completely optimum on critical examination. Addition of one of the organic stimulants to such a medium resulted in a slightly improved fermentation over that obtained when only phosphate was added (Fig. 3). Apparently, besides available phosphorus, some factors are missing from molasses. But the significance of these factors is so small that it is of no practical importance. The general conclusion of this investigation is, consequently, that sugar-beet molasses is an excellent raw material for the 2,3-butanediol fermentation, provided sufficient soluble phosphate salts are added.

It must be emphasized that the composition of various molasses is very variable. It may be that molasses like those of the non-Steffen variety can be used without the addition of any phosphate. The Chatham 1947 molasses, which was used throughout this work, had only 0.03% phosphorus as phosphorus pentoxide, which must be considered low for molasses. Freeman and Morrison (4) reported much higher amounts in the molasses that they used. Their high-test molasses contained about 0.06% and crude sugar-beet molasses as much as 0.1%. Nevertheless, these workers had to add some phosphate to their media for an optimum fermentation. Their molasses apparently contained more "total phosphorus pentoxide" but was low in "accessible phosphorus pentoxide".

Most of the graphs and tables in this paper point also to the phenomenon that stimulation of growth and fermentation by phosphate is most effective in the first stages of the fermentation. A prolonged incubation period camouflages the beneficial action of the phosphate, and sometimes the final yields in media without added phosphate are exactly the same as in those with added

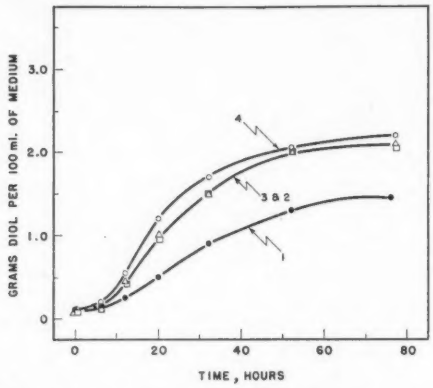

FIG. 3. *Yield of diol from B. polymyxa strain C42(3)E13 on a 10% molasses medium with added bran and dibasic sodium phosphate. Curves numbered: (1) Basal medium. (2) Basal medium plus 0.11% bran. (3) Basal medium plus dibasic sodium phosphate. (4) Basal medium plus dibasic sodium phosphate and bran.*

phosphate. This is characteristic of the effect of growth factors present in suboptimum amounts. Some of the growth factor concerned is used up and later excreted by the first-generation cells when they become senile or autolysed.

Acknowledgments

The authors wish to acknowledge the assistance rendered by Mr. A. K. Light in the chemical determination of phosphorus in molasses, bran, corn-steep liquor, and in the ashes of these substances; by Dr. D. Andrychuk in the spectroscopic analyses; and by Mr. F. Rollin who was responsible for much of the routine analytical work.

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A FRACTIONATING COLUMN TO PROVIDE WATER OF VARIOUS DISSOLVED OXYGEN CONTENT[1]

By F. E. J. Fry[2]

Abstract

A train is described through which water is cascaded downward while nitrogen passes slowly upward. In this the oxygen content of the water, which may be drawn off at various levels, is progressively decreased. The range of oxygen found in a typical run varies from 4.84 ml. per liter to 0.34 ml. per liter, there being 60 steps between these two levels.

The apparatus described below provides about 1 liter per minute of water containing various levels of dissolved oxygen. Tap water brought to the desired temperature passes down through a fractionating column while a slow flow of nitrogen passes upward.

The column consists of a 4 ft. length of 2 in. Pyrex tubing in which there is an initial reduction in the oxygen content of the water and a series of flasks from each of which in turn water of progressively lower oxygen content can be drawn. These flasks are of two types. For major steps in decreasing oxygen concentration, 1 liter flasks are used. When smaller steps are required, a train of 10 250 ml. Erlenmeyers made as indicated by sealing the neck of one into the bottom of the one above, is employed. The oxygen levels provided by the liter flasks are of the order of 30% apart, those provided by the Erlenmeyers differ by about 10%.

The 2 in. tube is provided with three openings along its side through which alternate water inlets are inserted. A two-hole stopper at the upper end accommodates the uppermost water inlet and a short length of glass tubing through which the gas escapes. The bottom of the tube is provided with a 1 in. neck which can be inserted through a one-holed stopper into the liter flasks or can be directly connected by tubing to the train of Erlenmeyers. The 2 in. tube is filled with glass marbles of approximately $\frac{1}{2}$ in. diameter.

The flasks are made from the 1 liter Florence type. At the upper side an opening is made to accommodate a No. 10 stopper. A 1 in. tube, $4\frac{1}{2}$ in. long, with the upper end closed over but provided with two series of four and six holes respectively, approximately $\frac{1}{4}$ in. in diameter, is sealed through the bottom, its free end projecting approximately $1\frac{3}{4}$ in. The uppermost series of holes serves for the upward passage of the nitrogen. The series about 1 in. lower allows the water to cascade to the next flask. A side arm of $\frac{1}{4}$ in. tubing is provided as indicated so that water may be drawn from the pool that gathers

[1] *Manuscript received September 26, 1950.*
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TABLE I

A TYPICAL SET OF LEVELS OF OXYGEN PROVIDED BY THE FRACTIONATING COLUMN. VALUES SUPPLIED BY M. P. SHEPARD. WATER TEMP. 10.5°C. TOTAL WATER FLOW (*a*) FIVE OUTLETS 1200 ML. PER MIN., (*b*) 10 OUTLETS 1440 ML. PER MIN. INITIAL OXYGEN CONTENT OF WATER 6.46–6.81 ML. PER LITER. THE LOWEST NUMBER INDICATES THE HIGHEST POSITION IN THE COLUMN

Inlet No.	Outlet number									
	1	2	3	4	5	6	7	8	9	10
(*a*) *Five outlet column*										
1	0.94	0.69	0.49	0.44	0.34					
2	1.46	0.91	0.74	0.57	0.43					
3	1.67	1.28	0.92	0.68	0.59					
4	3.50	2.76	1.96	1.44	1.24					
(*b*) *Ten outlet column*										
1	1.35	1.14	1.02	0.89	0.88	0.86	0.79	0.76	0.58	0.55
2	1.60	1.57	1.47	1.30	1.24	1.10	1.08	0.94	0.81	0.74
3	2.17	1.98	1.86	1.69	1.59	1.51	1.40	1.20	1.07	0.93
4	4.84	4.75	4.45	4.21	3.86	3.66	3.36	3.05	2.87	2.75

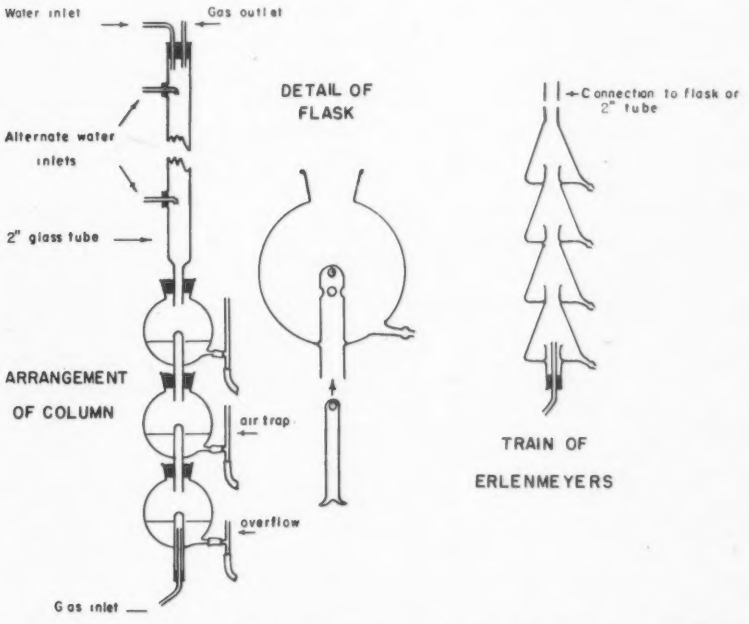

FIG. 1. *Sketch of apparatus, for details see text. The train of Erlenmeyers has been subsequently provided with test tube baffles as described for the larger flasks, the baffles being all linked together in this instance and suspended from a glass bar dropped into the top Erlenmeyer.*

in the flask. To prevent back pressure, the gas and water passages are separated in the neck by an inverted test tube of approximately $\frac{1}{2}$ in. outside diameter and 5 in. long suspended in the 1 in. tube by a loop of stainless steel wire. At low temperatures, the body of the flask is filled with glass marbles similar to those used in the 2 in. tube; at temperatures above 15°C., however, these are removed.

The train of flasks is set up as the diagram indicates. It has been found important to provide the outlet from each flask with a glass tee, as shown, to prevent air locks. The tee provided for the lowest flask has its upper arm short enough to operate as an overflow as well as an air trap. Some water is always allowed to go to waste through this overflow to maintain a seal in the apparatus.

Nitrogen is fed to the gas inlet at a rate of about 1 liter per minute. The flow of water from the column to the experimental chamber is conducted through rubber tubing and is regulated by a screw clamp placed at the point of greatest hydrostatic pressure.

This apparatus is currently being used in the determination of the lower levels of oxygen which can be tolerated by various species of fish. A description of the method of using the column for this purpose is given in (1). It should be noted that the column as used by Graham employed a train of large test tubes instead of the Erlenmeyers subsequently adopted. The performance of these two trains is essentially the same, but the Erlenmeyers are believed to be more convenient. It is most important to maintain the water temperature constant within close limits if the level of gas in the water delivered is to remain steady. Our apparatus was constructed by Mr. K. H. Chappell, 153 Roselawn Ave., Toronto.

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A STUDY OF CANADIAN MARGARINE WITH PARTICULAR REFERENCE TO ITS VITAMIN A CONTENT[1]

By T. K. Murray, J. H. Mahon, A. Wright,
R. A. Chapman, and J. A. Campbell

Abstract

Samples of margarine from 15 manufacturers have been analyzed for vitamin A, moisture, fat, curd, salt, ash, and antioxidants. The data indicate that the composition of margarine produced in Canada is relatively uniform. The average composition, in per cent, of 30 samples was: moisture 15.11, fat 80.70, curd 1.42, ash 2.96, and salt 2.91. The average vitamin A content of the product of 10 manufacturers producing 97.5% of the margarine made in Canada was found to be 4062 I.U. per 100 gm.

The ban on the sale of margarine in Canada was removed in January 1949. Since that time its production has increased until it now amounts to approximately one-third that of creamery butter. Enquiries received by the Food and Drug Laboratory have indicated an interest in its composition, uniformity, and, particularly, in its vitamin A content. It was decided that, in view of the importance of this product, a study should be made of all brands of margarine currently produced in Canada.

Thirty-three samples of margarine, the products of 15 manufacturers, were purchased by departmental inspectors during the months of April to August 1950 and analyzed for moisture, fat, curd, salt, ash, and vitamin A. Qualitative tests for antioxidants were also carried out on all samples.

Methods

The methods employed for the determination of moisture, fat, curd, salt, and ash were those recommended by the A.O.A.C. (1).

Moisture was determined by heating at 100°C. to constant weight.

Fat was extracted from the residue of the moisture determination with ether, and the remainder dried to constant weight.

Curd was determined by heating the residue from the fat determination at 500°C. The loss in weight represents curd.

Salt was washed from the margarine with hot water and titrated with standard silver nitrate.

Ash was determined by heating at 550°C. until the sample was carbon free. The values reported thus include salt.

Antioxidants

Tests for propyl gallate, butyl hydroxyanisole, nordihydroguaiaretic acid, and gum guaiacum were carried out by methods devised in this laboratory (5).

[1] *Manuscript received November 3, 1950.*
Contribution from the Food and Drug Division, Department of National Health and Welfare, Ottawa. Issued as paper No. 256 of the Canadian Committee on Food Preservation.

Vitamin A

It is generally agreed (3, 7) that the most reliable method for the estimation of vitamin A in margarine consists of measuring the absorption of a solution of margarine oils at 328 mμ, with a solution of unfortified margarine oils in the blank cell. The oils are separated by melting and filtering the margarine. Since the unfortified oils and the sample must be from the same batch, this method is practical only for those laboratories which have access to the margarine as it is being manufactured. Several methods have been proposed which do not require the use of unfortified oils. Wilkie (8) and Awapara *et al.* (2) have proposed methods in which the vitamin A is separated from interfering substances by chromatography. Other workers (7) have reported difficulty in obtaining reliable results by these methods. Neil and Luckman (6) have suggested destruction of the vitamin A by irradiation, after which the irrelevant absorption at 328 mμ is measured and subtracted from the total absorption of the sample at 328 mμ. Recently a method has been reported by Lodi (4), whereby the steroids, which interfere with the spectrophotometric determination of vitamin A, are removed by crystallization from cold methanol. Rice, Primm, and Coombes (7) have reported the results of a collaborative study involving five methods which show that the antimony trichloride method is probably the most reliable one for the determination of vitamin A in margarine when unfortified margarine oils are not available. This method involves the saponification of the whole margarine followed by extraction of the unsaponifiable fraction into ethyl ether. The antimony trichloride method has been used also by Goodwin and Morton (3).

As a result of preliminary work on several of the proposed methods, it was decided to test the reliability of the relatively simple method described by Rice, Primm, and Coombes (7) by means of an experiment in which the recovery of an added amount of vitamin A was measured. The margarine used in this experiment was complete in every way except that no vitamin A had been added during its manufacture. Weighed amounts of Canadian Reference Standard Vitamin A were added to four 10-gm. portions of this unfortified, but otherwise complete, margarine. The vitamin A was added at approximately the level found in fortified margarine and was then determined by the antimony trichloride method. The results summarized in Table I indicate that recoveries of vitamin A exceeded 95% in all cases. On this one sample of margarine the difference between the results by the increment procedure and by reference to the calibration curve was slight.

As a further check, the antimony trichloride method was compared with a spectrophotometric determination. Samples of finished margarine and the unfortified oils from which the margarine was manufactured were obtained directly from the manufacturer,* who also supplied the results of vitamin A determinations carried out in his laboratory. The spectrophotometric determinations were carried out on the separated margarine oil with a solution

* *Samples and results kindly supplied by Dr. H. W. Vahlteich, Best Foods Inc., Bayonne, N.J.*

TABLE I

RECOVERY OF VITAMIN A ADDED TO FOUR PORTIONS OF AN UNFORTIFIED MARGARINE

Reference standard added, I.U.	Vitamin A recovered as determined from			
	Calibration curve		Increment procedure	
	I.U.	%	I.U.	%
374	360	96	364	97
496	470	95	494	100
430	413	96	417	97
409	390	95	402	98

of the unfortified oil serving as a blank. The antimony trichloride method was applied to the whole margarine. In this case the blank consisted of solvent and reagent but did not contain the unfortified oil. The results shown in Table II indicate good agreement between methods although, as would be expected, the antimony trichloride values were somewhat higher than those determined by direct spectrophotometry. There was also good agreement between the results from the two laboratories.

TABLE II

COMPARISON OF THE VITAMIN A CONTENT OF A SAMPLE* OF MARGARINE AS DETERMINED BY TWO METHODS

Laboratory	Spectrophotometric I.U.	Antimony trichloride, I.U.	
		Calibration curve	Increment procedure
Best Foods*	17,900 17,700	18,800 18,900	
Food and Drug vitamin lab.	17,900	17,900 18,100 18,300 18,000	17,900 18,700 18,200

* *Sample and results kindly supplied by Dr. H. W. Vahlteich, Best Foods Inc., Bayonne, N.J.*

Since it appeared that the antimony trichloride method was capable of yielding reliable results, this method was used for the estimation of vitamin A in all samples reported herein. All values are the average of at least two separate saponifications. In most cases results were calculated by both the increment method and by direct reference to the calibration curve.

Results and Discussion

The detailed results of analysis of the margarine are shown in Table III and are, on the whole, within the range of the generally accepted composition of margarine. Average values in per cent for the following constituents were: moisture 15.11, fat 80.7, curd 1.42, ash 2.96, and salt 2.91. One sample was

found to be definitely low in fat and had a correspondingly high moisture content. The fact that four other samples from this manufacturer lacked the required amount of vitamin A probably reflects inadequate control of processing. Qualitative tests for the antioxidants propyl gallate, butyl hydroxyanisole, nordihydroguaiaretic acid, and gum guaiacum indicated that none of these was present.

TABLE III

COMPOSITION OF MARGARINES

Mfr.	Sample No.	Moisture, %	Fat, %	Curd, %	Ash, %	Salt, %	Vitamin A*, I.U./100 gm.
A	1	11.85	82.41	2.16	3.75	3.70	4259
	2	13.81	81.24	1.39	3.74	3.57	4565
B	1	14.03	81.60	1.46	3.08	3.03	4174
	2	15.07	80.86	1.34	3.05	3.04	3782
	3	14.95	80.74	1.55	3.11	3.03	3788
C	1	15.43	80.06	1.07	3.21	3.10	14114
	2	16.60	79.97	1.48	2.27	2.19	641
	3	—	—	—	—	—	3457
	4	—	—	—	—	—	3850
D	1	15.95	80.18	1.34	2.53	2.46	2626
	2	16.13	80.10	1.22	2.57	2.55	2606
E	1	13.62	81.81	1.62	2.92	2.82	4064
F	1	15.37	81.44	1.07	2.59	2.51	4330
G	1	15.33	82.43	0.60	2.00	1.99	4686
H	1	15.07	79.26	2.87	3.04	3.04	0
	2	15.59	79.98	1.75	3.04	2.92	950
	3	15.01	80.66	1.67	2.74	2.61	3535
	4	15.13	80.53	1.72	2.94	2.93	0
	5	25.46	70.76	1.69	2.24	2.23	2480
	6	—	—	—	—	—	3060
I	1	15.09	79.62	1.28	3.75	3.70	4136
	2	15.07	80.55	1.27	3.46	3.41	4275
	3	16.46	79.20	1.22	3.24	3.21	3710
	4	15.28	80.35	1.38	3.25	3.19	4253
J	1	15.20	80.69	1.18	3.24	3.20	3647
K	1	14.72	81.80	0.54	3.30	3.28	3816
L	1	13.18	82.59	1.51	3.14	2.98	3715
	2	15.02	80.74	1.36	3.02	2.99	3795
M	1	14.16	81.30	1.37	3.08	3.05	4101
	2	15.11	80.60	1.78	3.18	3.17	4027
N	1	14.61	82.00	1.56	2.26	2.25	977
	2	10.01	85.97	1.01	2.64	2.60	1500
O	1	15.11	81.58	1.09	2.57	2.48	1408

* *Labelled potency expressed as 3525 I.U./100 gm. or 16,000 I.U./lb. with the exception of G which is labelled at 4000 I.U./100 gm.*

The vitamin A results indicate that five of the companies whose products were tested manufactured margarine which was below labelled potency. Although this represents one-third of the companies which produce margarine, it would be erroneous to assume that 33% of the margarine marketed in Canada is deficient in vitamin A. Figures from the Dominion Bureau of Statistics show that for the two months during which the samples were purchased these five companies produced only approximately 2.5% of the margarine made in Canada. The average vitamin A content of the product of 10 manufacturers producing 97.5% of the margarine made in Canada was found to be 4062 International Units per 100 gm.

It may be concluded from this study that the composition of Canadian margarine is relatively uniform and that, over the period during which the samples were collected, only a very small percentage of the production was deficient in vitamin A.

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